Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 4030–4034, July 1983
Genetics

Activation of *Klebsiella pneumoniae* and *Rhizobium meliloti* nitrogenase promoters by *gln* (*ntr*) regulatory proteins

(nitrogen metabolism regulation/symbiotic nitrogen fixation/*lac* fusions)

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Communicated by Harold J. Evans, March 11, 1983

ABSTRACT We have studied the expression, in different *Escherichia coli gln* (*ntr*) mutants, of fusions (constructed *in vitro*) of the *nifHDK* (nitrogenase) promoters from *Klebsiella pneumoniae* and *Rhizobium meliloti* to *E. coli lacZ*. Derepression of the *K. pneumoniae nifH*::*lacZ* fusion requires the *glnF* (*ntrA*) gene product in addition to the *K. pneumoniae nifA* gene product, indicating that regulation of the *K. pneumoniae nif* genes is more closely integrated with the overall nitrogen control system than previously demonstrated. Derepression of the *R. meliloti nifH*::*lacZ* fusion in *E. coli* by the *K. pneumoniae nifA* gene product (which we had previously shown) exhibits the same requirement for *glnF*. Derepression of the *R. meliloti nifH*::*lacZ* fusion, but not the *K. pneumoniae nifH*::*lacZ* fusion, can be mediated by the *glnG* (*ntrC*) gene product, suggesting that the *gln* regulatory genes might directly regulate the symbiotic nitrogen fixation genes in *Rhizobium*.

The free-living nitrogen-fixing bacterium *Klebsiella pneumoniae* utilizes the enzyme nitrogenase to reduce N_2 to NH_4^+ under conditions of NH_4^+ starvation and low O_2 tension. Nitrogenase is composed of polypeptides encoded by genes *nifH*, *nifD*, and *nifK*, which are situated within an operon transcribed in the direction *nifH* to *nifK*. The *nifHDK* operon is itself located within a larger cluster of at least 17 contiguous *nif* genes, which are organized into seven or eight operons. One *nif* operon, the *nifLA* operon, codes for regulatory proteins (Fig. 1; reviewed in refs. 1 and 2). The *nifA* product is involved in activation of all the other *nif* operons, whereas the *nifL* product is involved in repression of these operons under certain physiological conditions (3, 4).

Recent studies of nitrogen assimilation in enteric bacteria have shown that the process is under the control of a central regulatory system. The products of three genes, *glnF* (or *ntrA*), *glnL* (or *ntrB*), and *glnG* (or *ntrC*), have been identified as the regulatory proteins involved in this process (refs. 6–9). Under conditions of nitrogen limitation, the *glnG* product appears to act in concert with the *glnF* product to activate a variety of nitrogen catabolism genes such as those involved in histidine utilization (*hut*) and proline utilization (*put*). Under conditions of nitrogen excess, the *glnG* product has been postulated to act in concert with the *glnL* product to repress the transcription of these same genes (reviewed in ref. 10). The *nif* genes of the enteric bacterium *K. pneumoniae* are indirectly under the control of the *gln* regulatory system due to the fact that the *nifLA* operon is regulated by *glnG* and *glnF* (11–13). Recently, our laboratory has shown that *nifA* can substitute for *glnG in vivo*; i.e., the *nifA* protein can activate the same genes as the *glnG* protein (13). In this study, we have examined whether the *gln* regulatory system can regulate *K. pneumoniae nif* genes in addition to the *nifLA* operon. We show that activation of a *nifH*::*lacZ* fusion (and by implication the *nifHDK* operon) requires *glnF* in addition to *nifA*.

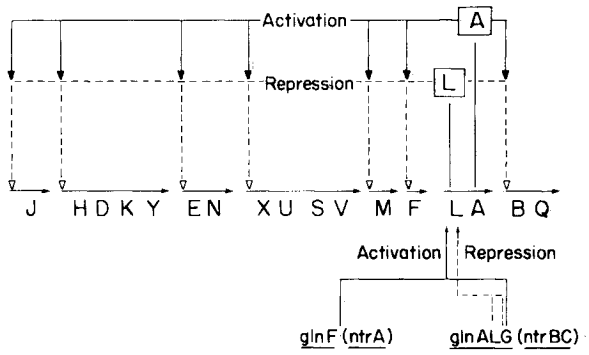

FIG. 1. The *nif* genes of *K. pneumoniae*, showing the transcription units and the current model of their regulation by the products of the *nifLA* operon (1–5).

In contrast to *K. pneumoniae*, the bacterium *Rhizobium meliloti* in the free-living state does not reduce dinitrogen; it reduces dinitrogen only in symbiosis with alfalfa. Our laboratory has shown that the *K. pneumoniae* and *R. meliloti nifHDK* genes are arranged similarly in an operon transcribed from *nifH* to *nifK* (14, 15) and that the *K. pneumoniae nifA* product can activate the promoters for the *nifHDK* operons from both *K. pneumoniae* and *R. meliloti* (16), indicating that the control systems regulating *nifHDK* expression might be conserved between the two genera. Here we examine the effect of *glnG* product on the expression in *Escherichia coli* of the *nifHDK* promoters from the two species. We now show that the *glnG* product can activate a *R. meliloti nifH*::*lacZ* fusion but not the *K. pneumoniae nifH*::*lacZ* fusion. This finding suggests that the *gln* regulatory proteins might be more directly involved in the regulation of *nif* genes in symbiotic nitrogen-fixing organisms than in *K. pneumoniae*.

MATERIALS AND METHODS

Construction of pVSA2. A 0.294-kilobase (kb) *Eco*RI/*Hga* I fragment containing the *nifH* promoter of *K. pneumoniae* (17) was inserted into the *lacZ*-carrying plasmid pMC1403 (18) as shown in Fig. 2*A*, so that the ATG start codon of *nifH* was in the same reading frame as the *lacZ* gene.

Construction of pVSP9. The 0.72-kb *Sal* I fragment containing the *R. meliloti nifH* promoter and the first 29 amino

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Abbreviation: kb, kilobase(s).

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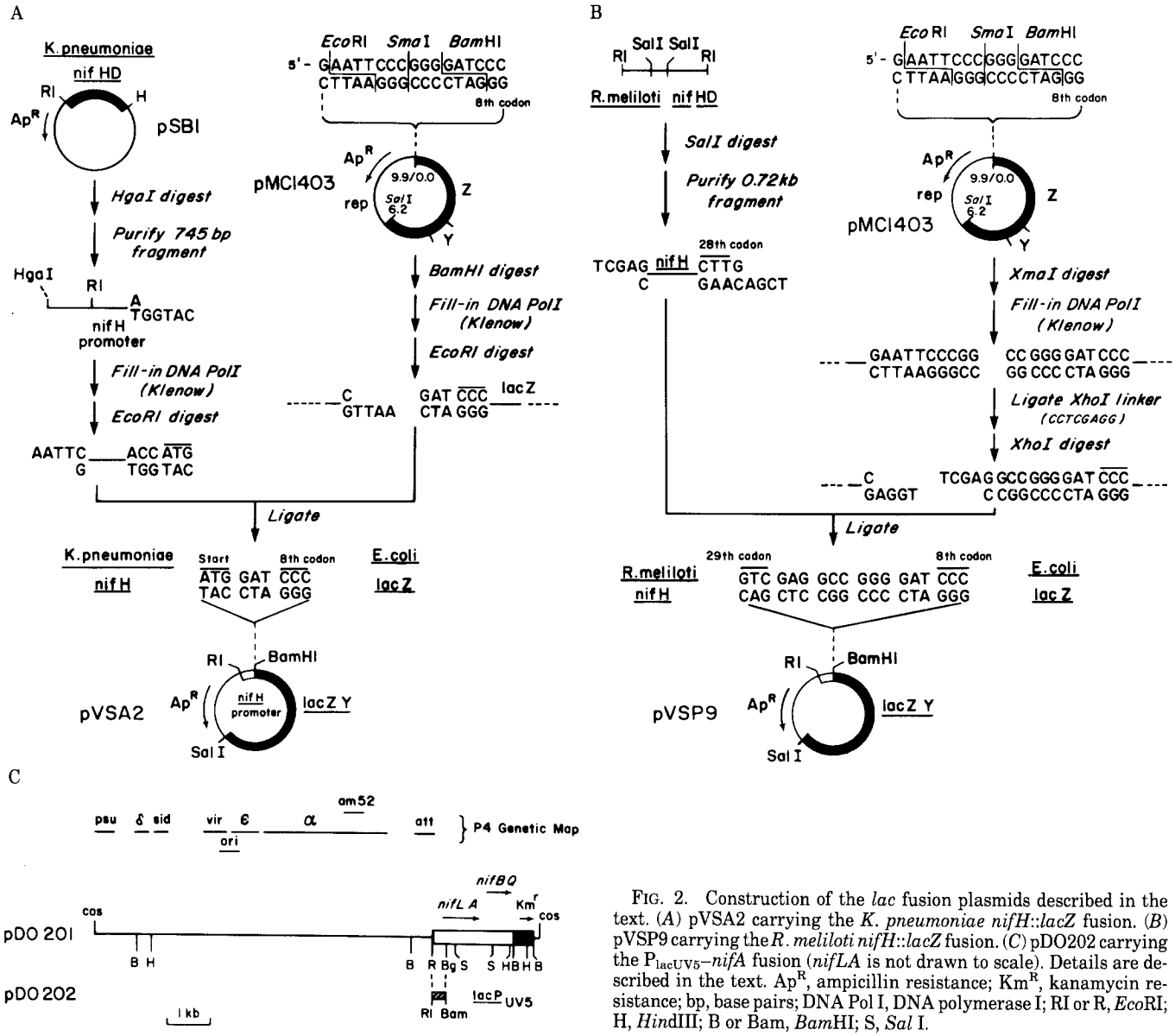

FIG. 2. Construction of the *lac* fusion plasmids described in the text. (*A*) pVSA2 carrying the *K. pneumoniae nifH*::*lacZ* fusion. (*B*) pVSP9 carrying the *R. meliloti nifH*::*lacZ* fusion. (*C*) pDO202 carrying the P_{lacUV5}–*nifA* fusion (*nifLA* is not drawn to scale). Details are described in the text. Ap^R, ampicillin resistance; Km^R, kanamycin resistance; bp, base pairs; DNA Pol I, DNA polymerase I; RI or R, *Eco*RI; H, *Hin*dIII; B or Bam, *Bam*HI; S, *Sal* I.

acids of *nifH* (19) was inserted into pMC1403 by using an *Xho* I linker (C-C-T-C-G-A-G-G; from Collaborative Research, Waltham, MA) as shown in Fig. 2*B*, generating an in-frame fusion of the 29th codon (GTC) of *R. meliloti nifH* to the 8th codon (CCC) of the *E. coli lacZ* gene. pVSA3 carries the *Eco*RI/*Sal* I fragment of pVSA2, which contains the *K. pneumoniae nifH*::*lacZY* fusion inserted into the tetracycline resistance gene of pDO105, a phage P4 vector (ref. 20; unpublished data); and pVSP9-1 carries the *Eco*RI/*Sal* I fragment of pVSP9, which contains the *R. meliloti nifH*::*lacZY* fusion, inserted into the tetracycline resistance gene of pDO105. pVSA3 and pVSP9-1 confer kanamycin resistance.

pDO516 is an ampicillin resistance plasmid carrying a transcriptional fusion of the *lac*UV5 promoter (P_{lacUV5}) to *nifA* on a pBR322 vector (13). Plasmid pPM517 carries the $lacI^Q$ gene inserted into pDO516 (P. McLean, personal communication). pDO201 carries the *nifLA* region cloned on a phage P4 vector that confers kanamycin resistance (Fig. 2*C*; unpublished data). pDO202 carries a P_{lacUV5}–*nifA* fusion obtained by replacing the 1.1-kb *Eco*RI/*Bgl* II fragment containing the NH_2-terminal region of *nifL* with a 0.1-kb *Eco*RI/*Bam*HI fragment containing the *lac*UV5 promoter (Fig. 2*C*).

pgln53Y (13) is an ampicillin-resistant tetracycline-sensitive derivative of pgln53 (9). Like pgln53, it is a $glnG^+$ plasmid that carries a fusion of the *glnA* promoter to *glnG* on a pBR322 vector; it synthesizes the *glnG* protein from the low-level-constitutive *glnA* promoter.

Measurement of β-Galactosidase Activity. Five-milliliter cultures of strains harboring *lacZ* fusions were grown anaerobically to saturation at 30°C in *nif*-derepression medium (3) with 0.2% $(NH_4)_2SO_4$ supplemented with 0.2% L-glutamine and the appropriate antibiotics (kanamycin at 10 μg/ml, ampicillin at 50 μg/ml, or both) and resuspended with nitrogen-rich or nitrogen-limiting media as indicated in Table 1 (with NH_4^+ and without NH_4^+, respectively). Nitrogen-limiting medium was *nif*-derepression medium containing antibiotics as above, but L-glutamine at only 100 μg/ml. Nitrogen-rich medium was nitrogen-limiting medium supplemented with 0.2% $(NH_4)_2SO_4$ and 0.2% L-glutamine. The cultures were incubated for 10 hr anaerobically at 30°C and centrifuged, and the β-galactosidase activity was determined as described by Miller (21).

Strains. *E. coli* YMC11 is *supE44* Δ*gln*(A–G)2000 Δ*lac*U169 (22); YMC10 is *supE44* Δ*lac*U169 (22); TH1 is *supE44* Δ*lac*U169 Δ*glnF* (from T. Hunt); DO1413 is *supE44* Δ*lac*U169 Δ*glnD* (13).

RESULTS

***nifA* Activation of *nifH* Promoters Requires *glnF*.** The *nifH*::*lacZ* fusions constructed as described in *Materials and Methods* were used to monitor the activity of the *nifH* promoters by measuring β-galactosidase activities in various *E. coli* strains containing different mutations in *gln* (*ntr*) regulatory genes. The *K. pneumoniae nifA* protein synthesized constitutively from a *lac* promoter on plasmid pDO202 activated the *K. pneumoniae nifH*::*lacZ* fusion on plasmid pVSA2 both in the absence and in the presence of NH_4^+ and glutamine (experiments 1-1 and 1-2, Table 1); this activation could also be carried out in a Δ*gln*(*ALG*) background (experiments 1-3 and 1-4), confirming previously published results that the *glnG* gene product is not necessary for activation by *nifA* (4). The lower levels of β-galactosidase measured in the presence of NH_4^+ could be due to the shorter half-life (50% of normal) of mRNA in cells growing on NH_4^+ (23). Because the decrease was more pronounced in a $glnL^+G^+$ strain, it is also possible that the *glnL* product could be mediating some repression of *nifH* in high concentrations of NH_4^+.

The *nifA*-dependent activation of the *K. pneumoniae nifH*::*lacZ* fusion was tested in the Δ*glnF* strain, TH1. It was clear from experiments 1-5 and 1-6 that *nifA* could not activate the *nifH*::*lacZ* fusion in this strain, either in the presence or in the absence of NH_4^+. This result was confirmed by using the *K. pneumoniae nifH*::*lacZ* fusion carried on a phage P4 vector and the P_{lacUV5}–*nifA* fusion carried on plasmid pPM517, which also carried a copy of the $lacI^Q$ gene to repress the *lac*UV5 promoter. Addition of isopropyl thiogalactoside induced synthesis of β-galactosidase in a $glnF^+$ strain (experiments 4-1 and 4-2), but not in a $glnF^-$ strain (experiments 4-3 and 4-4). Thus, in addition to *nifA*, *glnF* product appears to be required for activation of the *K. pneumoniae nifHDK* promoter.

We have shown elsewhere that the *K. pneumoniae* and *R. meliloti nifH* promoters are similar to the extent that the *R. meliloti nifH* promoter can be activated by the *K. pneumoniae nifA* product (16). This activation does not require the *glnG* product (ref. 16 and Table 1, experiments 1-7 and 1-8). When we repeated this experiment with the fusions in a $glnF^-$ background, we found that the *R. meliloti nifH*::*lacZ* fusion required both the *glnA* and *glnF* gene products (experiments 1-9 and 1-10), as was the case for activation of the *K. pneumoniae nifH*::*lacZ* fusion.

Table 1. β-Galactosidase activity in *nif–lac* fusion strains

Exp.	Strain	Relevant host genotype	Relevant plasmid properties*	β-Galactosidase, units: Without NH_4^+	β-Galactosidase, units: With NH_4^+
1-1	YMC10/pVSA2	gln^+	Kp *nifH*::*lacZ*	20	8
1-2	YMC10/pVSA2; pDO202	gln^+	Kp *nifH*::*lacZ*; P_{lacUV5}–*nifA*	3,073	1,830
1-3	YMC11/pVSA2; pDO201	Δ(*glnALG*)	Kp *nifH*::*lacZ*; Kp *nifLA*	58	73
1-4	YMC11/pVSA2; pDO202	Δ(*glnALG*)	Kp *nifH*::*lacZ*; P_{lacUV5}–*nifA*	4,226	3,306
1-5	TH1/pVSA2; pDO201	Δ(*glnF*)	Kp *nifH*::*lacZ*; Kp *nifLA*	12	10
1-6	TH1/pVSA2; pDO202	Δ(*glnF*)	Kp *nifH*::*lacZ*; P_{lacUV5}–*nifA*	14	10
1-7	YMC11/pVSP9; pDO201	Δ(*glnALG*)	Rm *nifH*::*lacZ*; Kp *nifLA*	55	49
1-8	YMC11/pVSP9; pDO202	Δ(*glnALG*)	Rm *nifH*::*lacZ*; P_{lacUV5}–*nifA*	1,721	1,968
1-9	TH1/pVSP9; pDO201	Δ(*glnF*)	Rm *nifH*::*lacZ*; Kp *nifLA*	110	98
1-10	TH1/pVSP9; pDO202	Δ(*glnF*)	Rm *nifH*::*lacZ*; P_{lacUV5}–*nifA*	111	107
1-11	YMC10/pVSP9	gln^+	Rm *nifH*::*lacZ*	436	49
1-12	YMC10/pVSP9; pDO202	gln^+	Rm *nifH*::*lacZ*; P_{lacUV5}–*nifA*	1,515	795
2-1	YMC10/pVSP9-1	gln^+	Rm *nifH*::*lacZ*	83	48
2-2	YMC11/pVSP9-1; pBR322	Δ(*glnALG*)	Rm *nifH*::*lacZ*; pBR322	21	18
2-3	YMC11/pVSP9-1; pgln53Y	Δ(*glnALG*)	Rm *nifH*::*lacZ*; P_{glnA}–*glnG*	278	209
2-4	YMC10/pVSA3	gln^+	Kp *nifH*::*lacZ*	10	9
2-5	YMC1/pVSA3; pBR322	Δ(*glnALG*)	Kp *nifH*::*lacZ*; pBR322	9	8
2-6	YMC11/pVSA3; pgln53Y	Δ(*glnALG*)	Kp *nifH*::*lacZ*; P_{glnA}–*glnG*	10	9
3-1	YMC10/pVSP9-1; pBR322	gln^+	Rm *nifH*::*lacZ*; pBR322	24	8
3-2	DO1413/pVSP9-1; pBR322	Δ(*glnD*)	Rm *nifH*::*lacZ*; pBR322	5	5
3-3	DO1413/pVSP9-1; pgln53Y	Δ(*glnD*)	Rm *nifH*::*lacZ*; P_{glnA}–*glnG*	41	34
3-4	YMC11/pVSP9-1; pBR322	Δ(*glnALG*)	Rm *nifH*::*lacZ*; pBR322	7	6
3-5	YMC11/pVSP9-1; pgln53Y	Δ(*glnALG*)	Rm *nifH*::*lacZ*; P_{glnA}–*glnG*	99	60
3-6	DO1413/pVSA3; pBR322	Δ(*glnD*)	Kp *nifH*::*lacZ*; pBR322	3	2
3-7	DO1413/pVSA3; pDO516	Δ(*glnD*)	Kp *nifH*::*lacZ*; P_{lacUV5}–*nifA*	270	264
3-8	DO1413/pVSA3; pgln53Y	Δ(*glnD*)	Kp *nifH*::*lacZ*; P_{glnA}–*glnG*	3	3
3-9	DP1413/pVSP9-1; pDO516	Δ(*glnD*)	Rm *nifH*::*lacZ*; P_{lacUV5}–*nifA*	155	137
4-1	YMC11/pVSA3; pPM517	Δ(*glnALG*)	Kp *nifH*::*lacZ*; $lacI^Q$ P_{lacUV5}–*nifA*	10	5
4-2	YMC11/pVSA3; pPM517 + isopropyl thiogalactoside	Δ(*glnALG*)	Kp *nifH*::*lacZ*; $lacI^Q$ P_{lacUV5}–*nifA*	192	149
4-3	TH1/pVSA3; pPM517	Δ(*glnF*)	Kp *nifH*::*lacZ*; $lacI^Q$ P_{lacUV5}–*nifA*	3	3
4-4	TH1/pVSA3; pPM517 + isopropyl thiogalactoside	Δ(*glnF*)	Kp *nifH*::*lacZ*; $lacI^Q$ P_{lacUV5}–*nifA*	3	3

Activation of the *K. pneumoniae* and *R. meliloti nifH*::*lacZ* fusions by *nifA*, *glnF*, and *glnG* gene products. See text for details. The experiments are divided into four sets, and the absolute values of β-galactosidase activity should be compared only within the same set. It is also necessary to keep in mind that the vectors carrying the *nifH*::*lacZ* fusions are not the same in all experiments—i.e., both pBR322 and phage P4 were used. The data in Exps. 1-3/1-4 and 1-7/1-8 are being published elsewhere (16) and are shown here only for comparison.

*Kp, *K. pneumoniae*; Rm, *R. meliloti*.

***glnG* Product Activates the *R. meliloti nifH* Promoter.** In a *glnG*$^+$ background, *nifA* protein activated the *R. meliloti nifH::lacZ* fusion (experiments 1-11 and 1-12); however, we observed derepression of the *R. meliloti nifH::lacZ* fusion even in the absence of *nifA*, when the cells were starved for NH_4^+ (experiment 1-11). On the other hand, no derepression occurred in a Δ*gln(ALG)* strain (experiments 1-7 and 1-8) or in a Δ*glnF* strain (experiments 1-9 and 1-10). It is likely, therefore, that *glnG* plus *glnF* products were activating *R. meliloti nifH* in the *glnG*$^+$ *E. coli* strain when the cells were under conditions of nitrogen deficiency.

The above results—i.e., the requirement for *glnF* for the activation of the *K. pneumoniae* and *R. meliloti nifH* promoters and the activation of the *R. meliloti nifH* promoter by the *glnG* product under derepressing conditions—were unexpected in the light of existing models of *nif* regulation (1, 2); they are, however, consistent with recent results from our laboratory demonstrating that *nifA* can function like *glnG* (13). We decided, therefore, to test directly whether *glnG* could replace *nifA* in the activation of *nifH::lacZ* fusions (as suggested by experiment 1-11). To do this we used a multicopy plasmid, pgln53Y, that carries the *glnG* gene fused to the *glnA* promoter; it does not carry intact *glnA* or *glnL* and synthesizes *glnG* product constitutively (9). Experiments 2-2 and 2-3 showed that the *glnG* product activated the *R. meliloti nifH::lacZ* fusion in an *E. coli* background. The activation of *R. meliloti nifH::lacZ* by multicopy *glnG* was of the same magnitude as that observed with multicopy *nifA* (not shown), but direct comparisons cannot be made without determining the amounts of *glnG* and *nifA* proteins present. In contrast, the *K. pneumoniae nifH::lacZ* fusion was not activated by *glnG* product, even when *glnG* product was being overproduced by the multicopy plasmid pgln53Y (experiments 2-5 and 2-6).

The experiments demonstrating *glnG* activation of *R. meliloti nifH::lacZ* (2-1 to 2-6), were repeated with the fusions in a *glnD*$^-$ background. The *glnD* product is involved in the derepression of the *glnALG* operon and *glnD*$^-$ mutants synthesize only low levels of *glnG* product (24). As expected, in a *glnD*$^-$ strain, the *R. meliloti nifH::lacZ* fusion was not derepressed upon NH_4^+ starvation (experiments 3-1 and 3-2). This effect was due to low levels of *glnG* product and not to a direct requirement for *glnD*; when the *glnG* constitutive plasmid, pgln53Y, was introduced into the same strain, activation was restored (experiment 3-3). However, the level of activation was lower than in a parallel experiment using a Δ(*glnALG*) strain (experiments 3-4 and 3-5), possibly due to repression mediated by *glnL* or non-uridylylated *glnB* product (9, 25). As before, we found that *glnG* could not activate the *K. pneumoniae nifH::lacZ* fusion (experiments 3-6 and 3-8). We also showed that the activation of the *R. meliloti* and *K. pneumoniae nifH::lacZ* fusions by *nifA* did not require *glnD* product (experiments 3-2 and 3-9, and experiments 3-6 and 3-7).

DISCUSSION

Role of *glnF* and *nifA*-Mediated Activation. The current model of *nif* regulation in *K. pneumoniae* can be summarized as follows: Under conditions of NH_4^+ starvation, transcription of *glnG* is activated and *glnG* gene product, in concert with the *glnF* gene product, activates transcription of the *nifLA* operon (reviewed in refs. 1, 2, and 5). The *nifA* gene product then activates all the other *nif* operons, which are also subject to repression by *nifL* under certain physiological conditions such as high O_2 tension (3). There has been no evidence presented so far that the proteins of the *gln* regulatory system interact with any *nif* operons other than *nifLA* (4). Rather, it has been proposed that *gln*-mediated regulation of *nif* expression is due solely to the action of *glnG* and *glnF* products on the *nifLA* promoter (3). Our demonstration that both *glnF* and *nifA* are required for activation of the *K. pneumoniae nifH::lacZ* and *R. meliloti nifH::lacZ* fusions is consistent with our previous discovery that *nifA* can substitute for *glnG* in activating a number of genes involved in nitrogen assimilation and with the model that *nifA* evolved from an ancestral *glnG* gene (13). Recently, Sibold and Elmerich (26) and Merrick (27) have also found that *glnF* is required for *nifHDK* expression even in the presence of a constitutive *nifA* plasmid.

The mode of action of *glnF* protein is not known at present. One possible mechanism that has been suggested is that it forms an activating complex with *glnG* (6); presumably it would function in the same manner in the case of *nifA*. It has also been proposed that *glnF* might be acting as the overall nitrogen sensor of the cell by converting *glnG* to an activator form during NH_4^+ starvation, either directly or indirectly; it might act indirectly by synthesizing a small effector molecule in response to nitrogen deficiency (6, 10). The latter model is by analogy to the adenylatcyclase–cAMP–cAMP-binding protein system involved in catabolite repression. Our data are not consistent with this model of *glnF* action for the following reasons: We find that (*i*) *nifA* absolutely requires *glnF* to activate the *nifH::lacZ* fusion, and (*ii*) when *nifA* is synthesized constitutively from a *lac* promoter in a *glnF*$^+$ background, it activates *nifH::lacZ* even in the presence of high levels of NH_4^+. If *glnF* were responding to NH_4^+ levels as proposed, activation under these conditions would not be expected. Our results are in agreement with those of Chen *et al.* (9), who have suggested that the regulatory responses of the cell to NH_4^+ starvation are mediated through *glnD* and *glnL*. While our results can be interpreted to suggest that *glnF* is always present in its active form, we cannot rule out some form of modulation of *glnF* activity or *glnF* product synthesis in response to changing NH_4^+ levels.

Differences Between *nifA* and *glnG*. If *nifA* and *glnG* products interact directly with the *nifH* promoters, our finding that either *nifA* or *glnG* can activate *R. meliloti nifH::lacZ* but that only *nifA* can activate *K. pneumoniae nifH::lacZ* suggests that the *nifA* and *glnG* gene products recognize different DNA sequences. In this light, it is interesting to compare the DNA sequences of the two *nifH* promoters (16) with the DNA sequence of the *nifLA* promoter (28); all three of these promoters can be activated by *nifA*. As illustrated in Fig. 3 (*i*) all three promoters share the sequence T-G-C-A in the −12 region; (*ii*) for the two promoters activated by *glnG* (*R. meliloti nifH* and *K. pneumoniae nifLA*), the homologous region at −12 is longer—i.e., T-T-T-G-C-A; (*iii*) in the case of the two *nifH* promoters, the homologous sequence at −12 is T-G-C-A-C, but there is also a longer 8-base-pair homologous sequence at −30 (A-C-G-G-C-T-G-G). Both of these *nifH* promoters show strong acti-

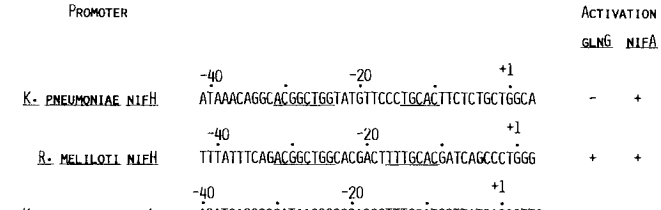

FIG. 3. Comparison of the promoter sequences of *K. pneumoniae nifH*, *R. meliloti nifH* (16), and *K. pneumoniae nifL* (28). The start points of transcription and the responses of the promoters to *nifA* and *glnG* gene products are indicated, and the homologous sequences at −12 and −30 are underlined.

vation with *nifA*. It is possible that the sequence T-G-C-A at −12 is a common element required by both *nifA* and *glnG* products for activation. However, the *glnG* product might require the complete sequence T-T-T-G-C-A, which is absent in the *K. pneumoniae nifH* promoter, explaining why this promoter cannot be activated by *glnG*. Recently, Beynon *et al.* have shown that a consensus sequence T-G-C-A is found at the same location in all the *K. pneumoniae nif* promoters; they suggest that this sequence is involved in RNA polymerase initiation complex formation in promoters that are active under nitrogen limitation conditions (J. L. Beynon, M. C. Cannon, V. Buchanon-Wollaston, and F. C. Cannon, personal communication).

The observation that the *K. pneumoniae nifH* promoter shows a high degree of specificity for *nifA* suggests that there has been an evolutionary selection for *K. pneumoniae* to develop a regulatory system that is highly specific for the *nif* gene cluster. Because nitrogen fixation is energy intensive, and because the nitrogenase enzyme is oxygen sensitive, it would be advantageous to keep *nif* genes repressed under aerobic conditions [a function provided by *nifL* (3)] while keeping other nitrogen-assimilation pathways open. Under such selection, a secondary regulatory circuit for indirect control of the *nif* genes by *glnLG* could have evolved.

In contrast to *K. pneumoniae*, the symbiotic reduction of N_2 by *Rhizobium* species may not be physiologically as stressful, because both the energy requirement and the O_2-protection system are supplied by the plant. Our finding that *glnG* can activate the *R. meliloti nifH* promoter raises the possibility that in *R. meliloti* the genes for nitrogenase are under the direct control of the *gln* regulatory proteins. [We should add the caution that an *E. coli* host was used for these experiments; however, the one *Rhizobium* RNA polymerase purified, from *R. leguminosarum*, does recognize the same phage T7 promoters as does *E. coli* RNA polymerase (29).] Such models have been proposed for *nif* regulation in *Rhizobium* "cowpea" sp. 32H1 (30). On the other hand, it is possible that *R. meliloti* does indeed have a "*nifA*-like" protein, but that it is intermediate in specificity between the *K. pneumoniae glnG* and *nifA* proteins. In this context we note that a putative regulatory gene closely linked to the *nifHDK* genes of *R. meliloti* has been recently identified in our laboratory (W. Szeto and L. Zimmerman, personal communication). A transposon insertion into this gene prevents synthesis of the products of all three nitrogenase structural genes (*nifHDK*) and in this respect it resembles *nifA*.

Like *K. pneumoniae*, bacteria in the genus *Azotobacter* fix nitrogen asymbiotically. In two species of *Azotobacter*, *nif* regulatory mutations that can be complemented by *K. pneumoniae nifA* have been found (31). It would be interesting to see if these can also be complemented by *glnG*. Such studies may clarify whether the evolution of a *nifA* protein, and of *nif* promoters that are specifically activated by it, is unique for *K. pneumoniae* or whether it is common among other organisms that fix nitrogen in the free-living state.

We thank T. Hunt for providing strain TH1, P. McLean for plasmid pPM517, and R. Hyde for typing the manuscript. This research was funded by National Science Foundation Grant PCM-8104193, awarded to F.M.A., and U.S. Department of Agriculture Grant 59-2253-1-1-722-0 awarded to W. J. Orme-Johnson at the Massachusetts Institute of Technology, with a subcontract to F.M.A.

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